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Antiviral Activity of Tobacco Smoke Condensate on Encephalomyocarditis Infection in Mice

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A water-soluble nontumorigenic acidic fraction of tobacco smoke condensate of cigarettes has been found to have antiviral activity against encephalomyocarditis (EMC) virus infection in mice. The portion of lower molecular weight was inhibitory to the growth of EMC virus, vesicular stomatitis virus, reovirus type 2, vaccinia virus, and poliovirus type 2, but not against adenovirus type 12, in KB cell cultures. The cigarette smoke agent did not induce serum interferon although it protected mice from EMC disease by pretreatment.

Through our antiviral screening work in the field of natural products of higher plant origin (2, 3), a water-soluble nonalkaloidal fraction of tobacco leaf was found to have antiviral activity against encephalomyocarditis (EMC) infection in mice (4). We now report that antiviral activity has also been found in a water-soluble nontumorigenic fraction of tobacco smoke condensate.

MATERIALS AND METHODS

Tobacco smoke condensate. A Kentucky reference cigarette smoke condensate (TSC) was supplied by T. S. Osdene and R. D. Carpenter, Philip Morris Research Center, Virginia.

Preparation of antiviral fractions of TSC. A 10-g amount of TSC was dissolved in 200 ml of chloroform and extracted in a separatory funnel with 1,600 ml of water made alkaline with 0.5 g of sodium carbonate. The aqueous portion, after flash concentration to 100 ml, was washed with 1,000 ml of fresh chloroform and adjusted to pH 8.0 with 1 N HCl; it was then filtered through an Amicon UM-10 membrane which separates substances with a molecular weight of 10,000. The filtrate and the retentate were designated Smoke-F and Smoke-R, respectively. Smoke-F showed only one peak, designated Smoke-F-G50, by further separation with a Sephadex G-50 column.

Separation of Swain's SA_w fraction (nontumorigenic) of TSC. Separation of the nontumorigenic water-soluble strong acid (SA_w) fraction was done by the method of Swain, Cooper, and Stedman (6). Briefly, 30 g of TSC was dissolved in 200 ml of ether and extracted with 300 ml of 1 N sodium hydroxide. The aqueous portion was repeatedly washed with fresh ether and chloroform to remove basic and neutral tumorigenic substances. After the pH of the aqueous portion had been adjusted to 6.1 with 6 N HCl, it was washed again with ether to remove weak acids (phenols, tumorigenic). The pH was again adjusted to 1.0 and the ether wash was repeated. The aqueous portion was flash-evaporated to dryness and absolute ethanol was added to separate salts. The ethanol-soluble portion was evaporated and the solid was dissolved in 100 ml of water. This water-soluble fraction was designated SA_w by Swain et al. (6) and was found to be nontumorigenic by Bock, Swain, and Stedman (1). The SA_w fraction was filtered through an Amicon UM-10 membrane (SA_w-F) or passed through a Sephadex G-50 column (SA_w-G50) to remove molecular substances over 10,000 (SA_w-R).

In vivo test. Agents were injected subcutaneously (sc) 3 h after or intraperitoneally (ip) 20 h before ip inoculation of an LD_{70-90} of EMC virus. Repeated or single doses were administered to Swiss albino mice weighing about 17 g. The animals were observed for 2 weeks.

In vitro test. Agents were added to the medium of KB cell cultures at the same time as or 1 h after virus inoculation. The viruses used were EMC, poliovirus type 2, vesicular stomatitis virus (VSV), reovirus type 2, vaccinia virus IHD strain, and adenovirus type 12; they had been passaged at least several times in KB cells before use. The medium (medium 199 with 2% calf serum) containing antiviral agents was changed every 2 days. The amount of virus and the degree of cytopathic effect (CPE) were recorded when they reached the maximum in the control cultures.

Titration of interferon in serum. Heart blood was collected 5 or 20 h after a single ip injection of agents and was diluted four times with medium 199; the serum was designated as the 1:4 dilution. This was the starting concentration for the assay of

interferon by the VSV-L cell system which has been described (4). As positive controls, polyinosinic-polycytidylic acid (poly I:C) was purchased from Microbiological Associates, Inc., and tilorone HCl was a gift from the Wm. S. Merrell Co.

RESULTS

Antiviral activity of TSC on EMC virus infection in mice. The therapeutic and prophylactic activities of TSC against EMC virus infection were tested by the established screening methods used for many years in this laboratory; the results are comparable with our previous data (2-4). Table 1 shows the results, including a comparison with two interferon inducers. Both low-molecular-weight Smoke-F and high-molecular-weight Smoke-R fractions, which were made by methods that removed total alkaloids and neutral substances from TSC, significantly reduced the mortality of EMC infection. Also, SA_w fraction and the low-molecular-weight portions (SA_w-G50 and -F) which were made by Swain's method to remove tumorigenic substances from TSC demonstrated definite antiviral activity. Thus, TSC contains an antiviral agent, although the prophylactic activity seemed to be weaker than that of standard interferon inducers (poly I:C and tilorone HCl).

Interferon assay in serum of mice treated with TSC. The prophylactic effect shown in Table 1 suggested that the TSC agent might be an interferon inducer, so the serum of the treated mice was titrated for interferon. The amount of interferon in the serum, collected 5 and 20 h after a single ip injection of agents, was measured. The results in Table 2 show that the three representative fractions of TSC did not induce measurable amounts of serum interferon, whereas poly I:C and tilorone induced definite amounts of interferon.

Antiviral activity of TSC on a single growth cycle of EMC virus in KB cells. From the negative results of serum interferon assay, further investigation of mode of action was directed to the tissue culture level. A system of EMC and KB cells which had previously been found to be refractory to the action of poly I:C and tilorone (4) was used. KB cells (1 ml of medium) were infected with a high multiplicity of EMC virus (100 $TCID_{100}$/cell), and 1 h later the cells were washed four times with Hanks solution. At various times postinfection, 2 mg (0.1 ml) of SA_w-G50 fraction was added, and the cells were incubated

TABLE 2. *Titration of interferon in serum of mice treated with tobacco smoke condensate and comparison with poly I:C and tilorone*

Agent	Dose (mg)[a]	Amt of serum-interferon[b]	
		5 h	20 h
Smoke-F	8	<4	<4
Smoke-R	5	<4	<4
SA_w-G50	4	<4	<4
Poly I:C	0.1	1,024	1,024
Tilorone	2	128	64

[a] All drugs were administered intraperitoneally.

[b] Reciprocal of the highest dilution of serum of which 0.1 ml clearly inhibited the cytopathic effect of VSV (100 TCD) in L cells (1 ml of medium) by pretreatment for 24 h.

TABLE 1. *Antiviral activity of tobacco smoke condensate on EMC infection in mice and comparison with poly I:C and tilorone HCl*

Agent	Treatment		No. died/total		Treated/control (%)	P
	Dose (mg)	Started (h)	Treated	Control		
Smoke-F	2	+3[a]	15/30	28/30	54	<.001
Smoke-F	8	−20[b]	47/90	78/90	60	<.001
Smoke-R	2	+3	14/30	28/30	50	<.001
Smoke-R	5	−20	9/20	17/20	53	<.005
SA_w	2	+3	15/30	26/30	58	<.001
SA_w	10	−20	25/50	45/50	56	<.001
SA_w-G50	4	−20	22/50	43/50	51	<.001
SA_w-F	8	−20	14/30	27/30	52	<.001
Poly I:C	0.02	+3	14/30	26/30	54	<.001
Poly I:C	0.02	−20	16/50	42/50	38	<.001
Tilorone HCl	1	−20	15/50	43/50	35	<.001

[a] Treatment was started 3 h after intraperitoneal infection and was administered subcutaneously twice daily for 5 days.

[b] Treatment was given once intraperitoneally 20 h before intraperitoneal infection.

for 15 h. The CPE at 15 h was recorded. In other tubes, the agent was added at the time of infection and removed 1 h later by repeated washing; then fresh medium without agent was added and the cells were incubated. It was found (Table 3) that the adsorption of virus onto the cells was not interfered with by the agent during the first hour of incubation; however, the intracellular viral process was suppressed before the appearance of CPE by the addition of the agent within 2 h after infection.

Antiviral activity of TSC on several viruses in KB cells. The antiviral spectrum of SA_w-G50 fraction in KB cells was determined (Table 4). The TSC agent was strongly suppressive against the CPE and growth of EMC virus, VSV, and vaccinia virus; it was moderately active against reovirus type 2 and poliovirus type 2, but was not active against adenovirus type 12.

TABLE 3. *Inhibitory effect of tobacco smoke condensate on the cytopathic effect of single-cycle growth of EMC virus in KB cells*[a]

Time postinfection when agent was added (h)	CPE at 15 h after infection
0[b]	4+
1	0
2	0
3	2+
4	4+
5	4+
6	4+
Control	4+

[a] The agent used was the SA_w-G50 fraction.

[b] The agent was added just after infection, incubated for 1 h, and then washed out. In the other experiments, the cells were washed 1 h postinfection, and the agent was added at the times indicated. In the control, no drug was added.

DISCUSSION

In a previous paper (4), we reported that the tobacco leaf of cigarettes contained an antiviral agent effective in mice and tissue cultures. Now it has been found that tobacco smoke condensate, collected by a smoking machine, possesses antiviral activity. Through the fractionation procedure which removes mainly alkaloids and chloroform-soluble neutral substances in TSC, the antiviral activity was found in the water-soluble fractions (Smoke-F and Smoke-R). Later, the same antiviral activity was found in the water-soluble strong acid fraction (SA_w) by the more advanced method of Swain et al. (6), which was adopted mainly for the separation of nontumorigenic and tumorigenic substances in TSC. According to the report of Bock et al. (1), the SA_w fraction seems not to contain tumorigenic substances; therefore, it may be suggested that the antiviral agent in the SA_w fraction is not one of the tumorigenic substances in TSC. The unit of the antiviral agent seems to be one of the low-molecular-weight substances in the fractions of Smoke-F, SA_w-G50, and SA_w-F, which might have a slight possibility of producing immunological hypersensitivity by repeated administration. The high-molecular-weight fractions (Smoke-R and SA_w-R) have been found to contain mainly the aggregated low-molecular-weight substances and a few higher-molecular-weight substances which could be separated by a Sephadex G-50 column and repeated ultrafiltrations (UM-10 membrane). Recently, the lower-molecular-weight SA_w-G50 fraction has been divided into three subfractions by the second pass in a Sephadex G-50 column. One of them (SA_w-G50-II), which represents only 10% of the solid of SA_w-G50, showed the same degree of anti-EMC activity in mice at a 0.4-mg dose as that of the original SA_w-G50 at 4

TABLE 4. *Antiviral activity of tobacco smoke condensate on several viruses in KB cell cultures*[a]

Virus	Inoculum size ($TCID_{100}$)	Incubation day	SA_w-G50-treated (1 mg/ml)		Control	
			CPE	$TCID_{100}$	CPE	$TCID_{100}$
EMC	10,000	1	0	10^4	4+	10^7
	100,000	1	1+		4+	
VSV	10,000	1	0	10^4	4+	10^7
	100,000	1	0	10^4	4+	10^7
Polio II	10,000	1	2+	10^6	4+	10^7
Reo II	10	4	1+	10^2	4+	10^4
Vaccinia	1,000	4	0	10	4+	10^6
Adeno 12	10	4	4+	10^2	4+	10^2

[a] The agent was added to the culture medium at the time of virus inoculation. Medium with drug was changed every 2 days. Incubation was stopped when CPE in controls reached maximum (4+).

mg. The other two dark-brown subfractions (90% of the solid of the original), which might be in a category of so-called "tobacco pigment" (5), were found to be toxic but not antiviral. The SA_w-G50-II fraction contains 4% peptides and 1% polysaccharides; the rest is unknown solids. The roughly estimated molecular weight is between 1,000 and 3,000. Further purification is in progress.

The fact that pretreatment with TSC agent for 20 h before EMC inoculation protected the mice from the fatal paralysis suggests that the mode of action might be interferon induction, but no serum interferon was detected. Furthermore, the TSC agent suppressed virus reproduction when added up to 2 or 3 h after infection in the single growth cycle experiment in KB cells which had been found to be defective in interferon production by poly I:C (4). Preliminary experiments also indicated that the agent had no enhancing action on humoral antibody production against sheep red blood cells in mice. Therefore, the antiviral action of TSC seems not to be via interferon and immunological defense systems but rather by the direct suppression of viral growth in the cells of mice.

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